

Expression of hypoxia-inducible factor-1 α , vascular endothelial growth factor and matrix metalloproteinase-2 in sacral chordomas

XIAOXIANG LI, ZHENWEI JI, YUNLEI MA, XIUCHUN QIU, QINGYU FAN and BAOAN MA

Department of Orthopedic Surgery, Orthopedics Oncology Institute of Chinese PLA, Tangdu Hospital, Fourth Military Medical University, Shaanxi 710038, P.R. China

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Abstract. Hypoxia-inducible factor-1 α (HIF-1 α) has been reported to transactivate the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2), which are frequently overexpressed in numerous types of cancer and are known to be significant regulators of angiogenesis. Few studies have investigated the role of these factors in solid tumors, particularly chordomas, which are rare tumors that are thought to originate from notochordal remnants. To clarify whether HIF-1 α is involved in angiogenesis in chordoma tissues, we examined the expression of HIF-1 α , VEGF and MMP-2 with immunohistochemistry using a tissue microarray containing 35 chordoma samples. The results indicated that HIF-1 α , VEGF and MMP-2 are expressed in the majority of chordoma samples. VEGF expression was significantly correlated with HIF-1 α and MMP-2 expression, as well as with microvessel density (MVD). However, the prognosis of the chordoma patients was not significantly associated with the expression of these factors, but was associated with MVD. The results therefore showed that there is a correlation between the expression of HIF-1 α , VEGF and MMP-2 in chordomas and that the angiogenic process is a potential therapeutic target for chordomas.

Introduction

Chordomas are rare tumors that are thought to originate from notochordal remnants. They arise from the sacrococcygeal region in approximately 50% of cases, from the sphenocapital area in 35% and from the mobile spine in 15% (1). More

males than females are affected by sacral chordoma; however, chordomas of the skull base appear to occur with equal frequency in males and females (2). Locally, these tumors are highly aggressive and frequently demonstrate local recurrence even following wide resection (3). However, there are no effective chemotherapeutic regimens available for the treatment of chordomas (4) and surgery remains the mainstay of chordoma management. Targeted molecular therapy has shown promising results in the treatment of malignancies with historically poor responses to chemotherapy. However, the expression of molecular markers in chordomas is not well understood. Thus, a better understanding of the molecular pathogenesis of this disease is required.

Hypoxia is a characteristic of all solid tumors. Under hypoxic conditions, tumor cells are deprived of oxygen due to a limited blood supply resulting from the abnormal tumor microvasculature (5). Hypoxia-inducible factor-1 α (HIF-1 α) is a transcription factor that regulates the pathways involved in tumor cell survival, proliferation, angiogenesis, invasion and metastasis. HIF-1 α is a significant protein that directly reacts to hypoxia (6). Hypoxia leads to a rapid increase in HIF-1 α protein levels, whereas HIF-1 α is maintained at low levels under normoxic conditions (7). HIF-1 α overexpression has been reported to correlate with tumor progression and an unfavorable prognosis in several types of cancer (8,9). In addition, HIF-1 α directly activates the expression of a number of pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) (10-12). Hypoxia-driven VEGF expression is considered to be a principal inducer of tumor angiogenesis. HIF-1 α regulates VEGF transcription by binding to the hypoxia response element (HRE) in the VEGF promoter region (13). HIF-1 α expression correlates with that of VEGF and microvessel density (MVD) in several types of tumor (14,15). Degradation and remodeling of the extracellular matrix (ECM) and basement membranes by proteolytic enzymes are essential steps in the processes of invasion and metastasis (16). MMP-2 has been shown to be one of the key enzymes regulating these processes. VEGF expression has also been found to correlate with MMP-2 expression in patients with gastric carcinoma (17).

In a previous study, we demonstrated that HIF-1 α is expressed in chordomas and is correlated with multidrug

Correspondence to: Dr Qingyu Fan, Department of Orthopedic Surgery, Orthopedics Oncology Institute of Chinese PLA, Tangdu Hospital, Fourth Military Medical University, No. 1 Xinsi Road, Xi'an, Shaanxi 710038, P.R. China
E-mail: bonetm@fmmu.edu.cn

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resistance-associated protein 1 (MRP1) (18). There are, however, no studies available concerning the association between HIF-1 α expression and clinicopathological findings in chordomas. The angiogenic process in sacral chordomas is also not fully understood. As a preliminary investigation of the molecular mechanisms of this rare disease, the aim of this study was to investigate the correlation between HIF-1 α , VEGF and MMP-2 expression and to clarify their clinicopathological significance in chordomas. Paraffin-embedded tissue samples from 35 sacral chordoma patients were retrospectively obtained. We examined the expression patterns of HIF-1 α , VEGF and MMP-2 by immunohistochemistry and staining was performed on a tissue microarray (TMA).

Materials and methods

Patients and tumor specimens. All cases of primary sacral chordoma diagnosed at Tangdu Hospital, The Fourth Military University (Shaanxi, China), between 1995 and 2010 were considered. The cases selected included patients with no history of chemotherapy or radiotherapy prior to surgery and a follow-up time of ≥ 12 months. A total of 35 patients diagnosed with sacral conventional chordoma were selected from the files. The patients ranged in age from 17 to 78 years (median, 53) and included 28 males and 7 females. The patient records were reviewed to collect the pathological data (tumor location and size and status of surgical resection margins), demographic data (patient age and gender) and follow-up information (time to local recurrence, distant metastasis and mortality) (Table I).

TMA's were prepared using a standard protocol (19). Archived chordoma tissues and representative hematoxylin and eosin (H&E) slides of each case were reviewed microscopically by a pathologist. The chordomas were identified on the corresponding H&E slides. Two tissue cores (2 mm in diameter) were removed from histologically identified representative regions of each formalin-fixed paraffin-embedded tumor. The study protocol was approved by the ethics committee of Tangdu Hospital. Informed consent was obtained from each patient in accordance with committee regulations.

Immunohistochemical staining for HIF-1 α , VEGF, MMP-2 and CD34. The TMA slides were investigated by immunohistochemistry for the expression of HIF-1 α (H1 α 67, dilution 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), VEGF (VG1, dilution 1:200; Dako, Copenhagen, Denmark) and MMP-2 (polyclonal, dilution 1:150; Bioss, Beijing, China) using the ChemMate™ Envision+HRP/DAB kit (Dako). The pre-treatment of sections with heat-induced epitope retrieval was performed according to the specification for each antibody. Negative controls were prepared using phosphate-buffered solution (PBS) instead of the primary antibody. The TMA slides were independently evaluated by two pathologists who had no prior knowledge of the clinical data or histological findings.

The immunohistochemical results for the HIF-1 α protein were then classified (-, no staining; +, nuclear staining in $<1\%$ of cells; ++, nuclear staining in 1-10% of cells and/or weak cytoplasmic staining; +++, nuclear staining in 11-50% of cells and/or distinct cytoplasmic staining; +++++, nuclear staining in $>50\%$ of cells and/or strong cytoplasmic staining) (20). For

statistical analyses, tumors with a final staining score of - or + with a weak or moderate/strong immunoreactivity were classified as the low expression group and were compared with tumors with scores of ++, +++ or +++++ that were classified as the high expression group (20). VEGF expression was defined as positive if distinct staining of the cytoplasm was observed in $\geq 10\%$ of tumor cells (14). Cytoplasmic MMP-2 staining was classified as: low expression exhibited staining in $<50\%$ of cells and high expression exhibited staining in $\geq 50\%$ of cells (21).

Evaluation of MVD. Paraffin sections (4 μ m) were cut from formalin-fixed paraffin-embedded tissue before tissue cores were removed for CD34 staining. CD34 (QEnd10, Ready to use, Dako) staining was used to assess MVD by light microscopy at the site of the highest number of capillaries and small venules. Highly vascular areas were identified by scanning tumor sections at a low power ($\times 100$). MVD was determined by observing the identified six fields for each sample at a magnification of $\times 400$, and the means were then calculated (14). A visible lumen was not necessary for a structure to be defined as a vessel (22).

Statistical analysis. Statistical analysis was performed using Microsoft Excel 2003 (Microsoft, Seattle, WA, USA) and SPSS Statistics 17.0 (SPSS, Chicago, IL, USA) for Windows. The significance of the observed associations was determined using the Mann-Whitney U test. The Spearman's rank correlation test was used to determine the association between HIF-1 α , VEGF and MMP-2. The impact of HIF-1 α , VEGF and MMP-2 expression on patient survival was assessed using the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Immunohistochemical staining of HIF-1 α , VEGF and MMP-2. HIF-1 α , VEGF and MMP-2 immunostaining was assessed and the samples were grouped into high- or low-grade categories. HIF-1 α expression showed a nuclear and/or cytoplasmic staining pattern with diverse intensities (Fig. 1). Of the 35 chordomas, 26 (74.3%) exhibited strongly positive HIF-1 α staining (score ++, +++ or +++) and 9 (25.7%) showed weakly positive staining (score - or +). Immunohistochemical staining of VEGF and MMP-2 was observed predominantly in the cytoplasm of the tumor cells. Of the 35 chordoma cases, 25 (71.4%) exhibited high VEGF expression levels. MMP-2 expression was classified as high in 24 (68.6%) of the chordoma cases. The immunohistochemical staining of each protein and their correlation with patient age, gender, tumor size, local recurrence and distant metastases are shown in Table II. No correlation was found between any of the three factors and clinicopathological characteristics (patient age, gender, tumor size, local recurrence and distant metastases) for any patient ($P > 0.05$).

Correlation between HIF-1 α , VEGF and MMP-2 expression. Of the 35 sacral chordoma samples, 26 (74.3%) showed intense HIF-1 α immunoreactivity in the nucleus and/or cytoplasm of the tumor cells. In the 26 sacral chordomas classified as having a high level of expression of HIF-1 α , the VEGF-positive and VEGF-negative expression frequency was 22/26

Table I. Clinicopathological characteristics of study cases.

No.	Age (years)	Gender	Size ^a (cm)	Margin status	Local recurrence (m)	Metastasis (location/m)	Outcome	Follow-up (m)
1	37	Female	10	NE	8	Lung/49	AWD	49
2	50	Male	5	-	No	No	NED	21
3	60	Male	5	NE	12	Lung/81	DD	86
4	56	Male	5	+	No	No	NED	18
5	53	Male	9	NE	No	No	NED	97
6	55	Male	8	NE	9	L3, abdomen/45	DD	51
7	47	Male	11	NE	34	No	NED	89
8	56	Male	8	+	No	No	NED	13
9	44	Female	6	-	25	No	NED	37
10	54	Male	5	NE	111	GM/111	DD	146
11	17	Male	9	+	20	T11-12/20	AWD	20
12	58	Male	4	NE	24	No	NED	29
13	77	Male	5	-	38	No	AWD	66
14	67	Male	7	NE	17	No	NED	30
15	49	Male	8	+	75	No	AWD	75
16	55	Male	7	-	5	No	NED	12
17	51	Female	6	+	61	No	DD	69
18	60	Female	5	NE	34	Abdomen/34	DD	81
19	51	Female	14	NE	41	No	DD	92
20	67	Male	8	+	7	No	AWD	38
21	47	Male	5	-	No	No	NED	43
22	52	Female	9	NE	23	No	AWD	23
23	27	Male	12	NE	25	No	AWD	25
24	74	Male	10	-	10	No	DD	43
25	78	Male	7	NE	6	No	DD	23
26	47	Male	4	-	No	No	NED	13
27	51	Male	8	NE	32	No	AWD	120
28	50	Male	9	-	No	No	NED	46
29	58	Female	8	NE	37	No	NED	65
30	59	Male	16	+	58	No	NED	63
31	62	Male	30	-	9	No	DD	112
32	56	Male	4	-	10	No	DD	39
33	50	Male	5	-	No	No	NED	73
34	53	Male	8	NE	No	No	NED	61
35	49	Male	6	NE	No	No	NED	45

AWD, alive with disease; DD, died of disease; NED, no evidence of disease; +, positive; -, negative; NE, not evaluable; GM, Gluteus maximus; m, months. ^aLargest diameter of tumor.

(84.6%) and 4/26 (15.4%), respectively. By contrast, of the 9 chordomas classified as exhibiting a low level of expression of HIF-1 α , 3/9 (33.3%) were VEGF-positive and 6/9 (66.7%) were VEGF-negative. The correlation between the HIF-1 α and VEGF expression levels was significant ($P=0.002$). In addition, of the 26 sacral chordomas classified as showing a high HIF-1 α expression, the frequencies of high and low MMP-2 expression were 20/26 (76.9%) and 6/26 (23.1%), respectively. There tended to be an association between the expression levels of HIF-1 α and MMP-2, but the correlation was not significant ($P=0.074$). The Spearman's rank correlation analysis indicated that the expression of VEGF was positively correlated with MMP-2 expression ($P=0.001$).

Correlation between the protein expression and MVD. The mean MVD value was 14.2 ± 4.0 . The expression levels of HIF-1 α , VEGF and MMP-2 compared with MVD are shown in Fig. 2. The mean MVD (in microvessels per field) in the high and low HIF-1 α expression groups was 14.4 ± 3.4 and 13.5 ± 5.5 , respectively. The mean MVD in the VEGF-positive and VEGF-negative groups was 15.3 ± 3.7 and 11.5 ± 3.6 , respectively, and the mean MVD in the high and low MMP-2 expression groups was 14.9 ± 4.0 and 12.6 ± 3.6 , respectively. VEGF expression was significantly associated with MVD ($P=0.008$), whereas the expression levels of HIF-1 α and MMP-2 were not significantly associated with MVD, as determined by the Mann-Whitney U test ($P=0.540$ and $P=0.092$, respectively).

Table II. Correlation of HIF-1 α , VEGF and MMP-2 expression with clinicopathological characteristics in chordoma cases.

Characteristics	Case number	HIF-1 α			VEGF			MMP-2		
		High	Low	P-value ^a	High	Low	P-value	High	Low	P-value
Age (years)										
>53 ^b	18	13	5	0.810	13	5	0.928	10	8	0.152
\leq 53	17	13	4		12	5		14	3	
Gender										
Female	7	7	0	0.239	6	1	0.529	5	2	0.903
Male	28	19	9		19	9		19	9	
Size of tumor (cm)										
<8 ^b	16	11	5	0.565	12	4	0.733	10	6	0.563
\geq 8	19	15	4		13	6		14	5	
Recurrence										
Yes	25	18	7	0.725	16	9	0.240	16	9	0.494
No	10	8	2		9	1		8	2	
Metastasis										
Yes	6	4	2	0.781	4	2	0.872	4	2	0.958
No	29	22	7		21	8		20	9	

^aMann-Whitney U test. ^bMedian. HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2.

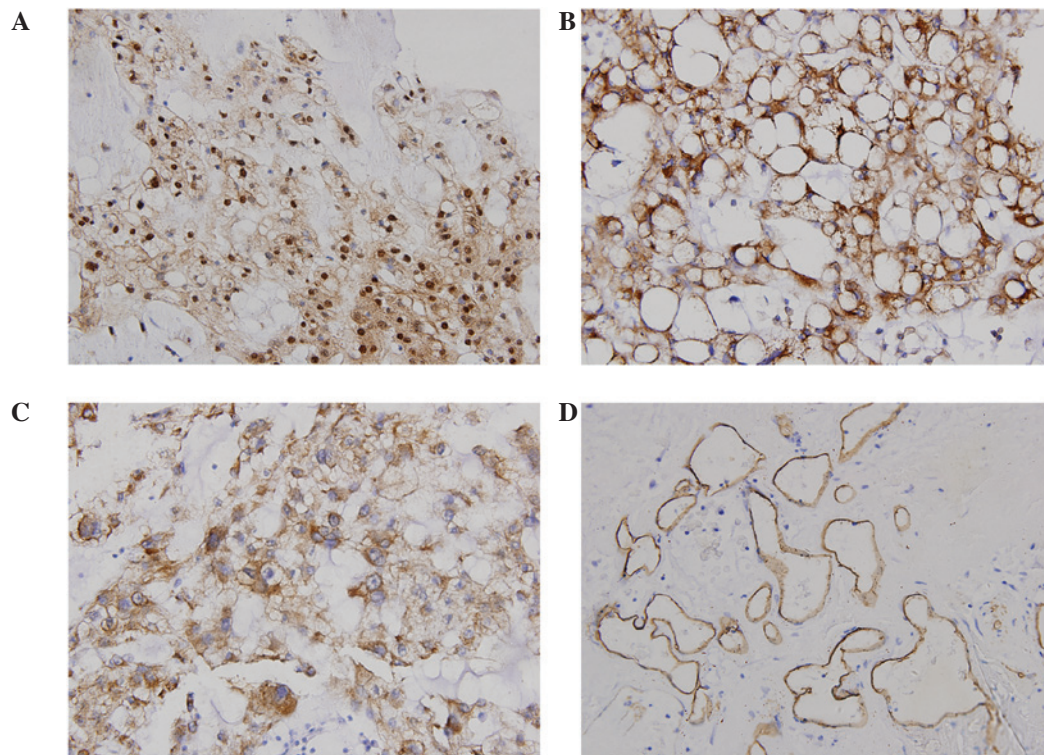


Figure 1. Expression of HIF-1 α , VEGF, MMP-2 and CD34 in primary chordoma samples is shown. (A) Strong HIF-1 α immunoreactivity in the cytoplasm and nuclei of tumor cells. (B) Positive immunohistochemical staining of VEGF. VEGF immunoreactivity is present in the cytoplasm of tumor cells. (C) MMP-2 was observed in the cytoplasm of tumor cells. (D) CD34 staining of chordoma tissue. HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2.

Correlation between patient survival and MVD and HIF-1 α , VEGF and MMP-2 expression. To investigate whether HIF-1 α , VEGF and MMP-2 expression levels are associated with the

outcome of patients with sacral chordoma, Kaplan-Meier analyses were performed. The mean duration of follow-up was 54.7 months (range, 12-146). Local recurrence occurred

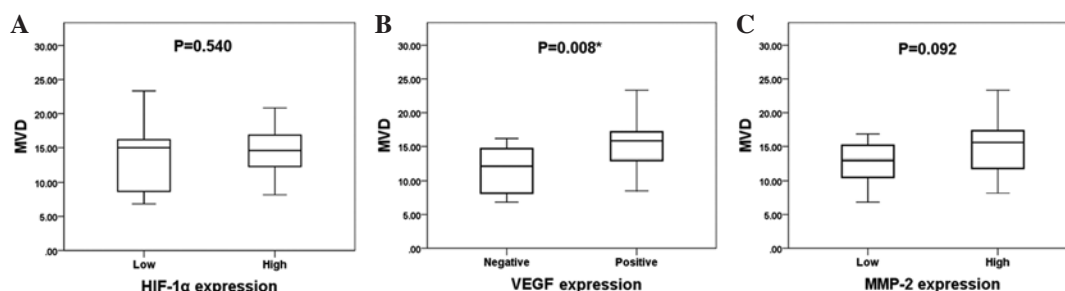


Figure 2. Correlation between MVD and the expression of (A) HIF-1 α , (B) VEGF and (C) MMP-2. The Mann-Whitney U test revealed that VEGF expression was significantly associated with MVD (B) ($P=0.008$). The expression levels of (A) HIF-1 α and (C) MMP-2 were not associated with MVD ($P=0.540$ and $P=0.092$, respectively). Statistically significant difference. HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2; MVD, microvessel density.

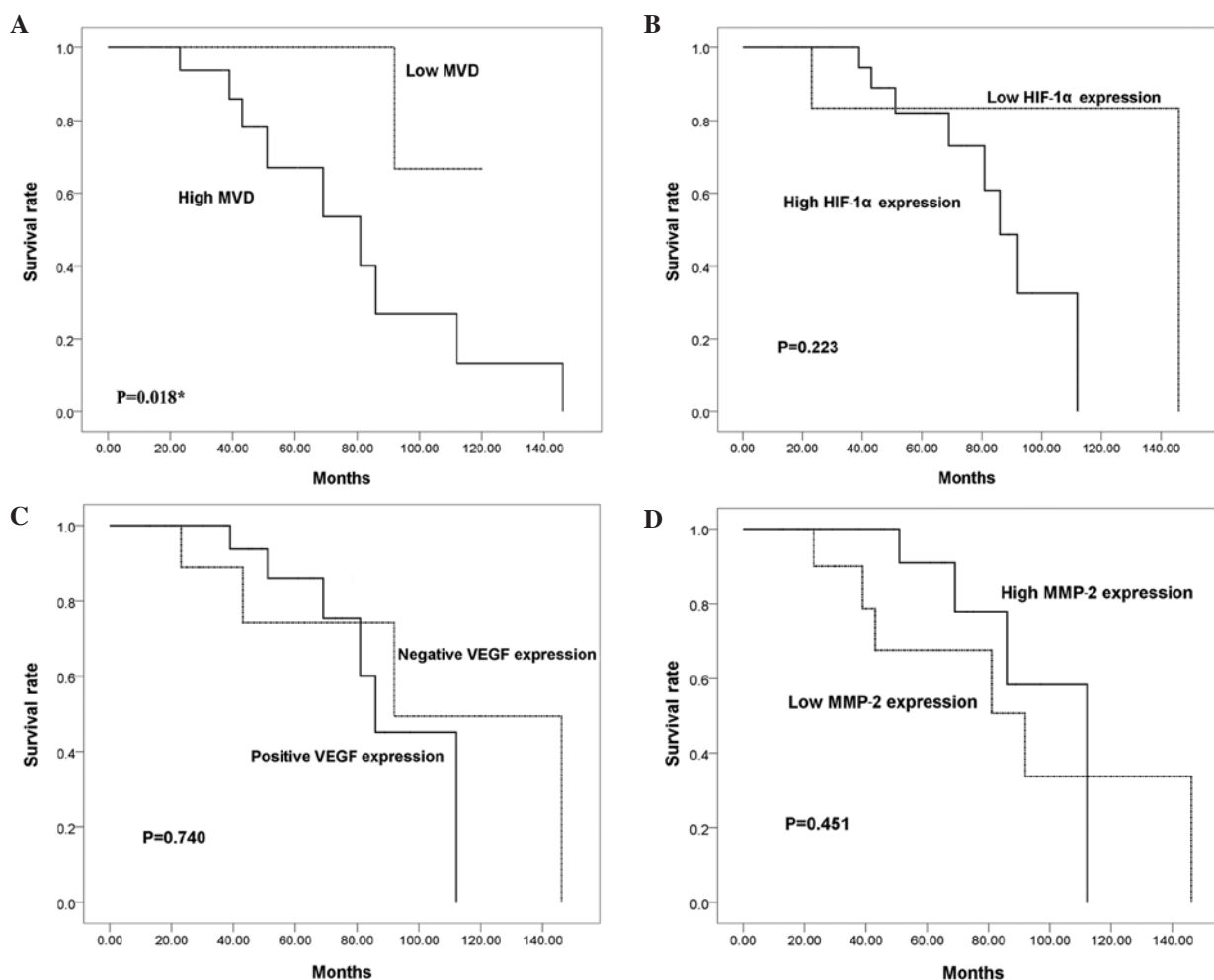


Figure 3. Impact of MVD and HIF-1 α , VEGF and MMP-2 expression on patient overall survival (log-rank test) is shown. (A) The log-rank test revealed a significantly shorter overall survival rate in patients with tumors with a high MVD ($P=0.018$). Kaplan-Meier analyses indicated that there were no significant differences in the survival rates between (B) the high and low HIF-1 α expression groups, (C) the VEGF-positive and VEGF-negative groups or (D) the high and low MMP-2 expression groups ($P=0.223$, $P=0.740$ and $P=0.451$, respectively). HIF-1 α , hypoxia-inducible factor-1 α ; VEGF, vascular endothelial growth factor; MMP-2, matrix metalloproteinase-2; MVD, microvessel density.

in 25 (71.4%) of the 35 chordoma patients during follow-up, with an average time to first local recurrence of 29.2 months (range, 5-111). Six patients presented with metastatic disease when the disease progressed to a more advanced stage (median time, 47 months; range, 20-111). The overall survival rates at 5 and 10 years were 82.3 and 29.9%, respectively. Kaplan-Meier analyses, however, indicated that there were no

significant differences in the survival rates between the high and low HIF-1 α expression groups, the VEGF-positive and VEGF-negative groups or the high and low MMP-2 expression groups ($P=0.223$, $P=0.740$ and $P=0.451$, respectively; Fig. 3). The survival of patients with a high MVD value was significantly shorter than that of patients with a low MVD value ($P=0.018$).

Discussion

Angiogenesis is an early event in carcinogenesis and one of the hallmark characteristics of tumors (23). Neovascularization is critical for tumor progression as the supply of oxygen and nutrients becomes limited in tumor cells located more than 100 μ m away from a blood vessel (24). Angiogenesis is a multistep process including basement membrane degradation, endothelial cell migration, sprouting into the interstitial space, endothelial cell proliferation, lumen formation and new basement membrane and anastomosis formation (25). There are several angiogenesis-related and -promoting factors that are thought to be essential for this process during tumor development. A compelling body of evidence indicates that VEGF is one of the most significant pro-angiogenic factors involved in tumor growth and metastasis. It is widely accepted that VEGF expression is mediated by HIF-1 α under hypoxic conditions (11,14,15,17). During hypoxia, HIF-1 α is protected from ubiquitination and proteasomal degradation and activates the expression of a number of pro-angiogenic factors, including VEGF, by binding to the HRE in the VEGF promoter region. Furthermore, HIF-1 α and VEGF expression levels have been analysed in a number of types of human cancer and their intracellular expression levels have been found to correlate with tumorigenesis and angiogenesis (14,15). Multiple proteinases, including MMPs, are also considered to be involved in the degradation of the vascular basement membrane and remodeling of the ECM during angiogenesis. Although the mechanisms underlying the HIF-1 α -mediated regulation of MMP-2 protein synthesis are currently unclear, it has been demonstrated that the overexpression of HIF-1 α may lead to an increase in the secretion and activation of MMP-2 (12). MMP-2 expression has also been reported to correlate with VEGF levels in patients diagnosed with gastric carcinoma (17), indicating that MMP-2 expression is closely linked with angiogenesis. However, the correlation between angiogenesis and chordomas is not well understood and has not been extensively studied. The present study was performed to retrospectively evaluate HIF-1 α , VEGF and MMP-2 immunohistochemical reactivity in sacral chordoma patients and to explore the correlation of the expression of these proteins with clinicopathological characteristics and prognosis. MVD was assessed using CD34 to determine its correlation with patient outcome.

In this study, we investigated HIF-1 α , VEGF and MMP-2 expression levels in human sacral chordoma using immunohistochemistry. Staining of HIF-1 α was observed in the cytoplasm and/or nuclei of tumor cells. However, we found that differences in clinicopathological characteristics, including patient age, gender, tumor size, local recurrence and distant metastasis, did not correlate with the expression levels of HIF-1 α , VEGF or MMP-2. Our results demonstrate that there is a significant correlation between HIF-1 α and VEGF expression levels. This study is the first to demonstrate such a correlation in human chordomas. It is also the first to explore the correlation between VEGF and MMP-2 immunoreactivity in chordomas. In addition, we have determined that the expression levels of MMP-2 are correlated with the expression of HIF-1 α , although this correlation was not significant. Previous studies have indicated that the three pro-angiogenic factors are associated with patient prognosis for several malignancies (17,20,21).

In our study, 35 cases with follow-up data were examined to determine whether the expression levels of HIF-1 α , VEGF and MMP-2 were of prognostic value. Chordoma patient outcome, however, was not significantly associated with the expression of the three proteins. We quantified tumor angiogenesis based on MVD using CD34 labeling. As a result, a significant correlation was found between the expression of VEGF and MVD. Furthermore, MVD was found to be associated with sacral chordoma patient prognosis. Chordomas are rare and the number of specimens was limited; there were only 35 samples in this study. As the group of low HIF-1 α expression had only 9 patients, it is likely that the number of samples was insufficient for significance to be determined. However, the 10-year survival rate in the high HIF-1 α expression group versus the low expression group was 0 versus 83.3% (Fig. 2B). As a result, a larger number of samples is required to confirm whether or not these protein expression levels correlate with prognosis.

Chen *et al* found that VEGF expression correlated with MMP-9 expression and was significantly higher in sacral chordoma tissue compared with adjacent normal tissue, although the difference in the disease-free survival rates between the VEGF-positive and VEGF-negative groups was not significant (26). The present study and that by Chen *et al* found no association between VEGF expression and disease-free survival or the survival rate; however, the number of patients in the two studies may not be sufficient to obtain prognostic information. Naka *et al* found that in skull base chordoma, MMP-9 was rarely expressed, but MMP-2 expression was frequently observed (27). These authors also found that MMP-2 expression was of prognostic value in non-skull base chordoma, but not in skull base chordoma (27,28). Although different results were observed in the skull base and non-skull base chordomas, together, these data indicate that the expression of VEGF and MMPs may be significant in chordoma progression.

At present, the effective treatment of chordomas is challenging. Even following wide surgical resection, the tumor frequently recurs. Chordomas have long been known to be resistant to chemotherapeutic drugs, presenting an obstacle that has to be overcome for the effective treatment of this disease. Due to its rare occurrence and a limited number of cell lines, little is known concerning the molecular basis of this disease. The results of our previous study suggest that both chordoma tissue and cell lines express HIF-1 α and that the expression of HIF-1 α is correlated with MRP1, which is known to be involved in hypoxia-induced chemoresistance (18). Taken together, these data indicate that HIF-1 α is a significant contributor to the process of angiogenesis and hypoxia-induced chemoresistance in chordoma cells. As shown in this study, angiogenesis is a significant process in sacral chordoma. The process of angiogenesis may be a potential therapeutic target in the treatment of chordomas.

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